CLINICAL STUDY

Vascular endothelial growth factor (VEGF) and its receptor VEGFR-2 are highly expressed in ovarian granulosa cell tumors

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Abstract

Objective: Ovarian granulosa cell tumors (GCTs) are hormonally active sex cord stromal tumors accounting for 3–5% of all ovarian cancers. These tumors are generally diagnosed at an early stage but there is a high risk of recurrence, associated with high mortality. Treatment of recurrent GCTs is difficult, and biologically targeted treatment modalities are lacking. GCTs are highly vascularized, and angiogenic factors most probably play a role in their pathology. Vascular endothelial growth factor (VEGF) is a key regulator of tumor angiogenesis, but in GCTs, the role of VEGF and its receptors VEGFR-1 (FLT1) and VEGFR-2 (KDR) remains largely unknown. Our objective is to study the expression of VEGF and its receptors in human GCTs.

Methods: We analyzed GCTs from 106 patients for the expressions of VEGF and its receptors utilizing tumor tissue microarray, tumor mRNA, and patient serum samples.

Results: We found that VEGF and its main biologically active receptor VEGFR-2 were highly expressed in primary and recurrent GCTs, when compared with normal granulosa-lutein cells. The expression of VEGF correlated positively to tumor microvessel density and to VEGFR-2 expression at the protein ($P < 0.05$) and mRNA ($P < 0.05$) levels. In contrast to VEGFR-2, the expression of VEGFR-1 was weak. Tumor VEGF protein expression was not prognostic for recurrence, however, we found high levels of circulating VEGF in the serum of patients with primary GCT.

Conclusions: The results suggest an important role of VEGF and VEGFR-2 in GCT pathology and support the possibility of applying novel VEGF- or VEGFR-2-targeted treatments to patients with GCT.

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Introduction

Ovarian granulosa cell tumors (GCTs) are sex cord stromal tumors, which account for 3–5% of all ovarian cancer (reviewed in (1)). GCTs are hormonally active tumors, producing estrogens and inhibins, and causing the characteristic hormonal signs and symptoms of the disease (2). GCTs are also highly vascularized and hemorrhagic and may present as large abdominal masses with acute abdominal pain (1). The median age of diagnosis in the adult subtype of GCT is 50–54 years, while the more uncommon juvenile subtype (5%) is diagnosed in children and adolescents (3, 4). Generally, GCTs are diagnosed at an early stage and are considered to have relatively good prognosis with more than 90% 5-year survival rate (1). These tumors have, however, a high risk of recurrence associated with high mortality (5, 6).

Vascular endothelial growth factor (VEGF) is a key regulator of physiological and pathological angiogenesis

(reviewed in (7)) and acts by binding to its two tyrosine kinase receptors VEGFR-1 (Fms-like kinase-1, FLT1) and VEGFR-2 (fetal liver kinase-1, FLK1, or kinase-insert domain receptor, KDR) expressed primarily in the endothelial cells (7). In the human ovary, VEGF is crucial for reproductive function, regulating follicular development, angiogenesis, and the development and maintenance of the corpus luteum (8, 9). VEGF is expressed in the granulosa cells of preovulatory and ovulatory follicles and most abundantly in the granulosa-lutein cells of the highly vascularized corpus luteum (10, 11). Both VEGFR-1 and VEGFR-2 are expressed in the granulosa-lutein cells in the corpus luteum (11, 12), and VEGFR-1 is also expressed in the granulosa cells of preovulatory follicles (11). The expression of the VEGFRs in malignant granulosa cells is undocumented.

VEGF regulates tumor angiogenesis and it is expressed in the majority of human tumors, including those of the ovary (13). Its expression correlates with

tumor malignancy, and encouraging results have been obtained with anti-VEGF therapy in a wide range of neoplastic diseases (14, 15). A humanized anti-VEGF antibody (bevacizumab) is now indicated as a first-line treatment for several metastatic cancers and has shown promise in the treatment of recurrent epithelial ovarian cancer (16). VEGFR targeting tyrosine kinase inhibitors are also being evaluated in phase I–III clinical trials (17, 18).

VEGF expression has recently been reported in a small study of GCT patients (19, 20), and a few case reports show beneficial effects of bevacizumab on recurrent GCT patients (20, 21). The expression of VEGF has, however, not been thoroughly evaluated in primary or recurrent GCTs, and the expression patterns of the VEGF receptors in GCTs remain unknown. We addressed these issues in this study of 106 GCT patients to elucidate the role of VEGF and its receptors in the biology of these ovarian tumors.

Materials and methods

Patient characteristics

The ethical committee of the Helsinki University Central Hospital and National Supervisory Authority for Welfare and Health approved this study. We identified 106 GCT patients with available tumor tissue samples diagnosed at the Helsinki University Central Hospital from 1965 to 2009 and collected the clinicopathological data of the patients. The diagnoses were re-evaluated by an experienced pathologist (R B) using immunohistochemical (IHC) markers to confirm the adult GCT diagnoses (22). All available freshly frozen

samples ($n=35$) were tested for the C134W mutation in FOXL2 (23–25), and 97% of the tumors were mutation positive. Tumor subtype, mitotic index, and nuclear atypia were defined by the pathologist (R B), as previously described (22). For controls, normal human ovaries were retrieved from three patients undergoing hysterectomy for benign indications.

The mean age at diagnosis was 51.5 years (range 19–87 years), and 60 patients (57%) were postmenopausal and 46 (43%) were premenopausal at the time of diagnosis. Of all patients, 30.2% ($n=32$) had a recurrent disease, with a mean follow-up time of 13.7 years (range 0.1–37.8 years). The Kaplan–Meier analysis of recurrence was performed on 76 patients with available tissue samples, with a mean follow-up time of 14.7 years (range 0.7–33.5 years). GCT patient and tumor sample characteristics are described in detail in Table 1.

Tumor tissue microarray

We constructed a tumor tissue microarray containing four cores from each tumor in duplicates as described previously (22, 26). Seventy-nine primary and 12 recurrent GCT samples were available for this tissue array and for consecutive IHC analysis.

Immunohistochemistry

Paraffin-embedded sections of the microarray and normal human ovaries were subjected to IHC for the expressions of VEGF (A-20, sc-152, Santa Cruz

Table 1 Patient and tumor sample characteristics. Mean age, menopause (MP) status, tumor size and stage are reported at the time of primary granulosa cell tumor diagnosis, i.e. the time of the primary tumor operation.

		Sample type (n)					
		IHC (91)		mRNA (35)		Serum (12)	
		P (79)	R (12)	P (30)	R (5)	P (7)	R (5)
Mean age, year (range)		51.8 (19–20)	44.7 (26–65)	52.5 (27–76)	39.8 (29–49)	56.4 (51–64)	39.8 (29–49)
MP status	Pre-MP	36	7	11	5	2	5
	Post MP	43	5	19	0	5	0
Stage	Stage I	70		26		7	
	Ia	49		19		4	
	Ib	1		0		0	
	Ic	20		7		3	
	Stage II	6		3		0	
	Stage III	3		1		0	
Tumor size	<10 cm	48	9	17	4	3	4
	>10 cm	31	3	13	1	4	1
Tumor subtype	Differentiated	55	3	19	3	3	3
	Sarcomatoid	24	9	11	2	4	2
Nuclear atypia	High	61	9	24	5	4	5
	Low	18	3	6	0	3	0
Mitotic index	High	59	7	21	4	1	4
	Low	20	5	9	1	6	1
Recurrence	No	59		25		7	
	Yes	20		5		0	

P, primary tumors; R, recurrent tumors; IHC, immunohistochemistry.

Biotechnology, Santa Cruz, CA, USA), VEGFR-1 (C17, sc-316, Santa Cruz Biotechnology), and VEGFR-2 (A-3, sc-6251, Santa Cruz Biotechnology) as previously described (22). In control experiments, nonimmune serum replaced the primary antibody. The intensity of staining was analyzed from the four cores of each tumor as a consensus of two researchers (A F and M A). The staining patterns of VEGF, VEGFR-1, and VEGFR-2 were homogenous, and the immunoreactivities of the tumor cells were compared with those of the granulosa-lutein cells of the normal ovary. The tumors were further divided into two groups with staining intensities being either 'high' or 'low', the latter group including also the tumors that remained negative. The blood vessels were visualized by staining for blood endothelial marker CD34 (mouse-anti-human CD34, M7165, DAKO, Glostrup, Denmark) and counted in duplicates per visual field from the four cores of each tumor. Tumor microvessel density (MVD) was graded as 'high' with ≥ 60 vessels per visual field and as 'low' with < 60 per visual field.

Quantitative real-time PCR

The RNA was isolated from freshly frozen tumor samples of 30 primary and 5 recurrent GCTs according to the manufacturer's instructions (Nucleospin RNA/Protein kit, catalog no. 740 933.250, Macherey-Nagel, Düren, Germany) and further purified with RNA purification kit (Nucleospin RNA Clean up kit, catalog no. 740 948.50). First-strand cDNA synthesis was performed according to the manufacturer's instructions from 0.8 μ g total RNA using SYBR Green RT-PCR reagents and random hexamers (Applied Biosystems, Foster City, CA, USA). The following primers were used for real-time PCR: for *VEGF*, forward 5'-TGCAGATTA-TGCGGATCAAACC, reverse 5'-TGCATTCACATTTGT-TGTGCTGTAG; for *VEGFR-2*, forward 5'-GGAAGCTC-CTGAAGATCTGT, reverse 5'-GAGGATATTTCTGTGCCGC; for *GAPDH* control, forward 5'-TCATTTCTGCTGATGA-CAACG, reverse 5'-TTACTCCTTGAGGCCATGT. Standard curve method was applied using purified mRNA from an established human GCT cell line (KGN) (27) as standard. All analyses were performed in triplicate with an ABI PRISM 7700 sequence detection system (Applied Biosystems) according to the manufacturer's instructions. Quantitative real-time PCR of VEGFR-1 was not conducted due to the minimal expression in semiquantitative PCR (data not shown).

Serum analyses

Serum samples were obtained from 12 patients at the time of diagnosis of the primary ($n=7$) or recurrent ($n=5$) GCT. Serum samples were prepared and stored at -80°C until analysis and were analyzed in duplicates with ELISA (R&D Systems, Minneapolis, MN, USA) according to the manufacturer's instructions.

According to the manufacturer, the mean serum VEGF in healthy subjects is 220 pg/ml and the range is 62–707 pg/ml.

Statistical analysis

Statistical analysis was performed with JMP software (JMP 7.0.1, SAS Institute Inc., Cary, NC, USA) and the possible correlations were tested with Student's *t*-test, χ^2 , and Fischer's exact test or simple regression when appropriate. The Kaplan–Meier analysis was performed according to the methodology, using the time from diagnosis to first recurrence as the end point. Log-rank test was applied to compare the differences between the groups. *P* value < 0.05 was considered statistically significant.

Results

VEGF and its receptors VEGFR-1 and VEGFR-2 are expressed in human GCTs

IHC staining for VEGF, VEGFR-1, VEGFR-2, and CD34 is illustrated in Fig. 1. The staining for VEGF was observed in the cytoplasm of tumor cells with even distribution across the tumor cells and also in the endothelial lining of tumor blood vessels (Fig. 1A, higher inset in A). Staining for VEGFR-1 was less pronounced in the tumor cell cytoplasm and membranes, and the expression was also detected in the tumor blood vessel walls (Fig. 1B, higher inset in B). VEGFR-2, however, was strongly expressed in GCT tumor cells, mostly on the tumor cell membranes (Fig. 1C), and also in the tumor blood vessel

Figure 1 VEGF and its receptors VEGFR-1 and VEGFR-2 are expressed in GCTs. The staining patterns of GCTs are shown for VEGF (A), VEGFR-1 (B), VEGFR-2 (C), and CD-34 (D). Note the expression of VEGF and VEGFR-2 in the tumor cells (A and C) and in the blood vessels (higher insets in A and C). Arrows indicate blood vessels in higher insets in (A–D). GL indicates granulosa-lutein cells in the corpus luteum of the normal human ovary (lower insets in A–C). Original magnification $\times 20$, scale bar 100 μm .

walls (Fig. 1C, higher inset in C). For comparison, normal human ovaries were stained for VEGF, VEGFR-1, and VEGFR-2 (lower insets in Fig. 1A–C). In accordance with previous findings (11, 12), VEGF and VEGFR-2 were expressed in the granulosa-lutein cells of the corpus luteum (lower insets in Fig. 1A and C). VEGFR-1 expression in the granulosa-lutein cells was, however, negligible (lower inset in Fig. 1B).

VEGF protein expression correlates with VEGFR-1 and VEGFR-2 protein expression, and tumor MVD

The intensities of staining in the tumor cells were evaluated in 79 primary and 12 recurrent GCTs, and the tumors were divided into high- and low-stained groups (Table 2). The intensity of staining in normal granulosa-lutein cells represented the low expression group for VEGF, VEGFR-1, and VEGFR-2. Of all tumors, 65 (74%) stained high for VEGF, whereas VEGF staining was low in 23 (26%) of the tumors. Only six (7%) tumors (five primary and one recurrent) were negative for VEGF. Staining for VEGFR-1 was generally weak (Table 2) with 82% of all tumors exhibiting low staining, and 44 (48%) tumors (40 primary and 4 recurrent) remained negative. The staining for VEGFR-2 was more intense, being high in 82 (93%) of the tumors and low in 6 (7%) of all tumors respectively (Table 2). Only one (1%) primary tumor was negative for VEGFR-2. The number of microvessels varied from 6 to 171 (mean 45) per visual field (Table 2). There were no statistically significant differences in the staining patterns of primary and recurrent tumors. In all GCTs, the expression of VEGFR-1 and VEGFR-2 protein correlated positively to that of VEGF ($P < 0.05$; Table 3). The correlation between VEGFR-1 and VEGFR-2 expressions did not reach statistical significance probably due to small numbers in the different groups (Table 2). We also found that tumor MVD correlated positively to VEGF expression as analyzed in all tumors (Table 3).

Table 2 VEGF protein expression correlates positively to the expressions of VEGFR-1, VEGFR-2, and to tumor MVD. The intensity of staining and MVD were divided into groups with high or low expression. Note that high expression was seen in 74% of the tumors for VEGF, 82% for VEGFR-2, and only 16% for VEGFR-1. There were no statistical differences between the expression levels of primary and recurrent tumors.

		Tumors (n)		
		P (n=79)	R (n=12)	Tot (n=91)
VEGF	High	56 (73)	9 (82)	65 (74)
	Low	21 (27)	2 (18)	23 (26)
VEGFR-1	High	11 (14)	5 (45)	16 (18)
	Low	66 (86)	6 (55)	72 (82)
VEGFR-2	High	71 (92)	11 (100)	82 (93)
	Low	6 (8)	0 (0)	6 (7)
MVD	High	19 (24)	6 (50)	25 (28)
	Low	60 (76)	6 (50)	66 (72)

P, primary; R, recurrent; Tot, all tumors.

Table 3 Correlations of protein expressions in all GCTs. Note the positive correlation of VEGF expression to the expressions of VEGFR-1 and VEGFR-2, and to MVD.

	VEGF	VEGFR-1	VEGFR-2	MVD
VEGF		+	+	+
VEGFR-1	+		NS	NS
VEGFR-2	+	NS		NS
MVD	+	NS	NS	

+, positive correlation with $P < 0.05$; NS, not significant.

VEGF protein expression is not prognostic for recurrence

In the primary tumors, we could not find any correlations between the expressions of VEGF, VEGFR-1, or VEGFR-2 and primary tumor characteristics (tumor stage, subtype, tumor size, nuclear atypia, and mitotic index) or patient characteristics (age at diagnosis and menopausal status). Neither were there any correlations between VEGF, VEGFR-1, or VEGFR-2 expressions and tumor characteristics (tumor size, subtype, nuclear atypia, and mitotic index) in the recurrent tumors. When all GCTs were analyzed, there was, however, a positive correlation between tumor MVD and tumor mitotic index ($P < 0.05$), but not between MVD and other tumor characteristics. We also analyzed whether VEGF protein expression in the primary tumor could be prognostic for the recurrence in patients with GCT. The time to first recurrence is shown by the Kaplan–Meier analysis as to primary tumor VEGF expression in 76 patients (Fig. 2). There were 12 recurrences in the high VEGF group ($n = 55$) and 6 in the low VEGF-expressing group ($n = 21$) and the recurrence probability was similar in these groups.

VEGF and VEGFR-2 mRNA expressions correlate positively in primary and recurrent GCTs

We next analyzed the mRNA levels of VEGF and VEGFR-2 with quantitative real-time PCR in 35 GCTs (30 primary and 5 recurrent). We found that VEGF and VEGFR-2 mRNA expressions were expressed in the primary and recurrent GCTs (Fig. 3A and B), without any statistical difference between the primary and recurrent GCTs. The mRNA expression of VEGF correlated positively to that of VEGFR-2 (Fig. 3C). The mRNA expression of VEGF or VEGFR-2 in the primary or recurrent tumors did not show significant correlations to tumor or patient characteristics.

Circulating VEGF is present in high quantities in the serum of patients with primary GCT

We evaluated the serum levels of VEGF from seven primary and five recurrent GCT patients. At the time of diagnosis, the mean serum VEGF levels tended to be

Figure 2 VEGF protein expression is not prognostic for recurrence. The Kaplan–Meier curves for recurrence in the high (red line, $n=55$) and low (blue line, $n=21$) VEGF expression groups. The recurrence probability was similar between the groups.

higher in primary GCT patients (mean 557 pg/ml, median 411 pg/ml, range 107–1020 pg/ml) than in recurrent GCT patients (mean 219 pg/ml, median 149 pg/ml, range 40–539 pg/ml; Fig. 4), although there was no statistical difference between these groups ($P=0.097$). The serum VEGF levels did not correlate to the tumor size or stage, and there was no statistical difference in the tumor size between the primary and recurrent tumors.

Discussion

Tumor angiogenesis is a critical step in cancer progression, and anti-angiogenic cancer treatments have been extensively studied over the past decade. VEGF, being one of the main pro-angiogenic growth factors in many cancers, was the first to be targeted and anti-VEGF treatments are now in wide clinical use (15). Recently, the VEGF receptors have also shown promise as targets for anticancer drugs (28, 29). So far, little has been known about the expression of VEGF and its receptors in GCTs. We show that VEGF and its receptors are expressed in both primary and recurrent GCTs, and these findings provide the biological basis for the development and implementation of biologically targeted treatments to patients with GCT.

In accordance with previous findings (19, 20), we found that VEGF was abundantly expressed in GCTs, with almost all tumors (93%) staining positive for VEGF. VEGF was expressed homogenously in the cytoplasm of the tumor cells and also in the blood vessel endothelium. The expression of VEGF protein was higher in GCTs than in the nonmalignant granulosa cells,

indicating a role for VEGF in these tumors. The expression levels were similar in primary and recurrent GCTs, both at the protein and mRNA levels. Although we found no correlations between VEGF expression and tumor aggressiveness, the fact that both primary and recurrent tumors express VEGF at high levels suggests an important role of VEGF in the GCT pathology.

VEGFR-2, the main mediator of VEGF function, is usually expressed only in the blood vessel endothelial cells (30). We found that compared with normal granulosa-lutein cells, VEGFR-2 protein is highly expressed in the granulosa tumor cells. In normal endothelial cells, VEGF exposure leads to the down-regulation of the VEGFRs (31). This regulatory function is, however, lost in VEGFR-2-expressing cancer cells (32), and the previous findings from ovarian (33) and breast (34) cancer cells suggest a survival-promoting VEGF–VEGFR-2 autoloop, which can be inhibited with anti-VEGF treatment (bevacizumab) (35). In our analysis, the expression of VEGFR-2 protein and mRNA correlated with those of VEGF, implicating an autocrine role of VEGF also in GCTs. Furthermore, VEGFR-2 expression did not correlate to tumor MVD, reflecting the strong expression in the tumor cells, and further indicating that the role of VEGFR-2 in GCTs may be independent of the tumor vasculature. Although VEGFR-2 expression did not correlate to tumor aggressiveness, the remarkably high expression suggests a role of VEGF–VEGFR-2 signaling in GCTs that is worth further studies.

In contrast to VEGFR-2, the expression of VEGFR-1 in GCTs was low. Furthermore, VEGFR-1 is usually considered to function as a decoy receptor for VEGF, and it is not markedly phosphorylated upon VEGF binding (36). The prognostic role of VEGFR-1 in tumors has remained controversial (37, 38). In the search for novel anticancer treatments, some of the new small-molecule tyrosine kinase inhibitors are, however,

Figure 3 VEGF mRNA expression correlates positively to VEGFR-2 mRNA expression. Quantitative real-time PCR was performed on the mRNA isolated from 30 primary (P in A and B) and 5 recurrent (R in A and B) GCTs. Note the similar expression of VEGF and VEGFR-2 in primary and recurrent GCTs, and the positive correlation of VEGF mRNA expression to that of VEGFR-2 in primary and recurrent tumors (C).

Figure 4 High level of VEGF is present in the serum of patients with primary GCT. VEGF was measured at the time of diagnosis from the serum of patients with primary ($n=7$) and recurrent ($n=5$) GCT. The mean VEGF tended to be higher in the primary GCT samples than in the recurrent GCT samples, although the difference was not statistically significant ($P=0.097$).

designed also to inhibit VEGFR-1 (17). We found high VEGFR-1 protein expression in a minority of primary GCTs, and there was no correlation between VEGFR-1 expression and tumor aggressiveness.

As an indirect indicator of tumor angiogenesis, MVD has been shown to be of prognostic significance in ovarian cancer (39), but its role in GCTs is still unknown (40). Previous data suggest that mitotic index is a prognostic marker in GCTs (41–43), but this has not been found in all studies (22, 40). We found that VEGF protein expression correlated positively to tumor MVD, independent of tumor size or stage. High tumor MVD also correlated to high tumor mitotic index, implicating enhanced angiogenesis in the tumors with an active growth. These results implicate a role of VEGF-driven angiogenesis in the growing GCT and offer a possibility to implement biological adjuvant treatments on the actively growing GCTs with high mitotic index, even regardless of tumor size or stage.

It was of interest to evaluate whether VEGF and its receptors can be used in delineating the prognosis of GCT patients. We found no correlations between primary tumor VEGF, VEGFR-1, and VEGFR-2 protein or mRNA expressions and recurrence, and neither did primary tumor VEGF expression predict recurrence-free survival. Thus, based on this study, these parameters are not likely to be of value in the prognostic evaluation of GCT patients. In our analysis, circulating mean VEGF levels in primary tumor patients tended to be higher than in patients with recurrent disease. This may be due to increased VEGF producing tumor burden in primary compared with recurrent GCTs. The mean circulating

VEGF in primary GCT patients is similar to that observed in epithelial ovarian cancer patients (44, 45), but the range of circulating VEGF is wide, even in healthy subjects (46). A larger study of GCT patients and healthy control subjects needs to be studied to evaluate the clinical value of serum VEGF in these patients.

Surgery is the first-line treatment in primary GCTs, but the treatment of recurrent GCTs consists of a combination of surgery and chemotherapy. Recurrences are rather common (reaching 30% in this study) and often present in locations where surgical removal is not feasible (5, 47). Despite the developments in conventional chemotherapeutic combination treatments, mortality in recurrent GCT is high (5, 6), and thus the most challenges rise in the treatment of recurrent GCTs. Recently, anti-VEGF (bevacizumab) monotherapy was shown to be highly effective in a retrospective study of eight patients with recurrent GCTs (20). In addition, markedly reduced ascites formation was reported with bevacizumab treatment in a patient with recurrent GCT (21), reflecting the role of tumor-derived VEGF in the formation of cancer-related ascites (48, 49). Considering our findings on the high expression of VEGF and VEGFR-2 also in recurrent GCTs, these patients could benefit from the introduction of VEGF- or VEGFR-2-targeted anticancer drugs, in combination with traditional treatments. To test this, however, large multicenter clinical trials are required.

In conclusion, our findings implicate a role of VEGF and its receptor VEGFR-2 in GCT pathogenesis and support the targeting of VEGF and VEGFR-2 in the validation of new treatments for GCT patients.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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